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14. ABSTRACT Cellular, molecular and behavioral experiments were conducted in rats to determine the molecular basis of a widespread chronic pain that is frequently reported in veterans of the 1991 Gulf War. Behavioral studies examined whether chronic exposure to insecticides (chlorpyrifos, permethrin) and a nerve gas prophylactic (pyridostigmine bromide) could produce a delayed and persistent myalgia and arthralgia. Following a 60 day exposure to these Gulf War chemicals, we observed an increase in resting behaviors that persisted for 12 weeks after exposures had ceased. Muscle and vascular nociceptive neurons that were harvested from these animals, at 8 and 12 weeks post-exposure, exhibited broad alterations in the physiology of K _v ion channels (K _v 7 and other K _{DR} channel proteins) and Na _v 1.9 that were consistent with increased cellular excitability at the 12 week observation period. An imbalance between K _v 7 and Na _v 1.9 could prove to be the basis for a chronic pain condition sourced from a vulnerable subset of vascular and muscle nociceptors. Because these ion channels are widely expressed throughout the nervous system, maladaptations in their physiology could contribute to the development of a variety of sensory, motor, cognitive and autonomic symptoms associated with Gulf War Illness.					
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1. Introduction

Many veterans of the 1991 Persian Gulf War returned from that conflict with symptoms of chronic pain. Twice as many developed a widespread deep tissue pain following their return to the states (Kroenke et al., 1998). The type of symptoms that were reported were distinct from those associated with polytrauma, CRPS or NBI pains that can develop from load bearing microinjuries. The Research Advisory Committee on Gulf War Illness (GWI) has determined that pesticides may have contributed to the development of the symptoms of GWI (Binns et al., 2008; RAC2014). Our laboratory initiated a series of investigation to determine whether a subset of the many pesticides and related GW chemicals that were used during the conflict could produce chronic pain behaviors in a rat model; and whether there were molecular correlates of this pain present in ion channel proteins expressed in families of deep tissue nociceptors. The identification of persistent molecular maladaptations could provide targets for treatment. In the progress report that follows, we present findings from examination of how pesticides and a nerve gas prophylactic affects the long term physiology of K_v channels in afferent neurons. K_v channels play a fundamental role in the control of neural excitability and axonal coding throughout the nervous system. Because the veterans report a variety of deep tissue pains, our studies focused on nociceptors innervating muscle and blood vessels.

2. **Keywords:** pain, pesticides, pyridostigmine bromide, K_v channels, Gulf War Illness

3. Overall Project Summary

Muscle and joint pain are common symptoms associated with GWI (Haley Syndrome 3; Haley et al., 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). The Research Advisory Committee on Gulf War Illness (GWI) has determined that pesticides may have contributed to the development of the symptoms of GWI (Binns et al., 2008; RAC2014). During the brief course of the Gulf War, veterans were potentially exposed to 67 insecticides and repellants containing 37 distinct active ingredients (DoD Environmental Exposure Report: Pesticides, 2003; Binns et al., 2008).

Our laboratory has identified molecular adaptations in pain system neurons that result from exposure to GWI suspected pesticide neurotoxicants (permethrin, chlorpyrifos) and pyridostigmine bromide (Jiang et al., 2013; Nutter et al., 2013; Nutter and Cooper, 2014). These chemicals have direct interactions with important proteins expressed in the peripheral pain (nociceptor) system. Permethrin acutely modifies the physiology of central and peripheral nervous system Na^+ channels that are essential for pain coding ($\text{Na}_v1.6$, $\text{Na}_v1.7$, $\text{Na}_v1.8$, $\text{Na}_v1.9$; Soderlund et al., 2002; Bradberry et al., 2005; Ray and Fry, 2006; Nutter et al., 2013; Nutter and Cooper, 2014). Chronic exposure to anti-cholinesterases chlorpyrifos and PB (pyridostigmine bromide) upregulate expression of muscarinic acetylcholine receptor proteins that couple to important voltage sensitive K^+ channels that control neuronal excitability (K_v7 ; Abou-Donia et al., 2002; Abou-Donia et al., 2003; Abou-Donia et al., 2004). Following an 8 week exposure to a combination of PB, permethrin and chlorpyrifos (rats), we have demonstrated persistent modulation of neural channel proteins $\text{Na}_v1.9$ and K_v7 (Nutter et al., 2013; Nutter and Cooper, 2014). These molecular adaptations were documented 8 weeks after neurotoxicant exposure had ceased. Although those studies focused on the peripheral pain system, neuronal channel proteins $\text{Na}_v1.9$ and K_v7 have widespread representation that includes the hippocampus, neocortex, autonomic and enteric nervous systems where their dysregulation may contribute to the fuller spectrum of GWI cognitive, motor and autonomic symptoms (Jeong et al., 2000; Blum et al., 2002; Rugiero et al., 2003; Padilla et al., 2007; Brown and Passmore, 2009; Binns et al., 2008; Haley et al., 2013). The K_v7 family of ion channel proteins belong to a larger superfamily of K_{DR} proteins that control neural excitability in multiple ways. In the last year of the research

program, we examined how other members of the K_{DR} superfamily may have been affected by long term exposure to pesticides.

Objectives:

- 1) Develop an animal model of GW chronic pain.
- 2) Characterize molecular changes that occur in nociceptors following exposure to pesticides and pyridostigmine bromide
- 3) Characterize vascular afferents

Results:

Behavioral Studies

Task 5: To examine the development, time course and persistence of a myalgia in rats exposed to combinations of neurotoxicants (permethrin and chlorpyrifos) and pyridostigmine bromide

In previous studies funded by GWIRP, we reported persistent changes in key neuronal channel proteins following chronic exposure to GW chemicals, these changes did not appear in parallel with measurable behavioral indices of pain (Nutter et al., 2013; Nutter and Cooper, 2014). As part of the SOW (Task 5), we sought to create an exposure protocol that would induce a long lasting pain condition and subsequently determine whether K^+ channel neuronal proteins (K_{DR} , K_v7) exhibited properties consistent with a chronic pain condition.

In order to induce a behavioral effect, the frequency of NTPB dosing (permethrin, chlorpyrifos, pyridostigmine bromide) was increased. In prior studies supported by GWIRP, we treated rats with permethrin (topical; 2.6 mg/kg; mixture of 26.4% cis and 71.7% trans), chlorpyrifos, (subcutaneous; 120 mg/kg) and PB (oral gavage; 13 mg/kg). Treatments lasted 60 days, where permethrin was applied daily, PB was given daily for 15 days, in each of two 30 day periods, and

chlorpyrifos was injected once every 2 weeks (Nutter and Cooper, 2014). These doses and application protocols generally produced behavioral indices of pain only during the exposure periods (figure 1; from Nutter and Cooper, 2014). Any changes in pain behaviors occurring during exposure quickly returned to normal at the end of the exposure (Jiang et al., 2013; Nutter et al., 2014). Accordingly, we increased the frequency of the exposure ('intensified protocol') while retaining the same dose levels noted above. In separate groups of rats Permethrin and PB were given every day for 30 or 60 days, and chlorpyrifos was injected once every week in both the 30 and 60 day groups (figure 2).

Animals receiving the intensified dose protocol for 30 days exhibited some significant increases in resting for about 8 weeks after exposure. Thereafter, all measures were comparable to controls. No other measures were affected in the 30 day exposure group (figure 1). As shown in figure 2, increasing the frequency of dosing for 60 days produced long lasting behavioral changes; however the changes still fell short of convincing evidence of a chronic pain condition brought about by GW chemicals. Pain/pressure-withdrawal thresholds remained stable over the entire 21 week period of testing (figure 2A). Animals treated with higher frequency NTPB for 60 days did exhibit a long lasting increase in rest periods that persisted out to 12 weeks following the exposure (figure 2D). Animals in pain generally increase rest periods, and such behavior would be consistent with joint and muscle pain due to NTPB. However, despite an increase in rest periods, general activity (movement distance) was unchanged by NTPB (figure 2B). Moreover, when rats were active during the 15 minute test period, NTPB treated animals tended to move greater distances in the post exposure periods; and in particular, during movement activity they moved significantly faster than the vehicle treated animals at 8 weeks post exposure (figure 2C). By 12 weeks post-exposure the movement rate was comparable to controls, as was the total distance moved (figure 2B and 2C); resting was still significantly elevated at post-12 weeks. While the increase in the frequency of NTPB exposure for 60 days succeeded in producing some long lasting behavioral effects (resting), an encouraging outcome, the pattern of these effects did not seem totally consistent with a chronic pain condition. Our parallel molecular studies on these same animals examined whether the intensified NTPB treatment produced molecular adaptations that could lead to a chronic pain condition.

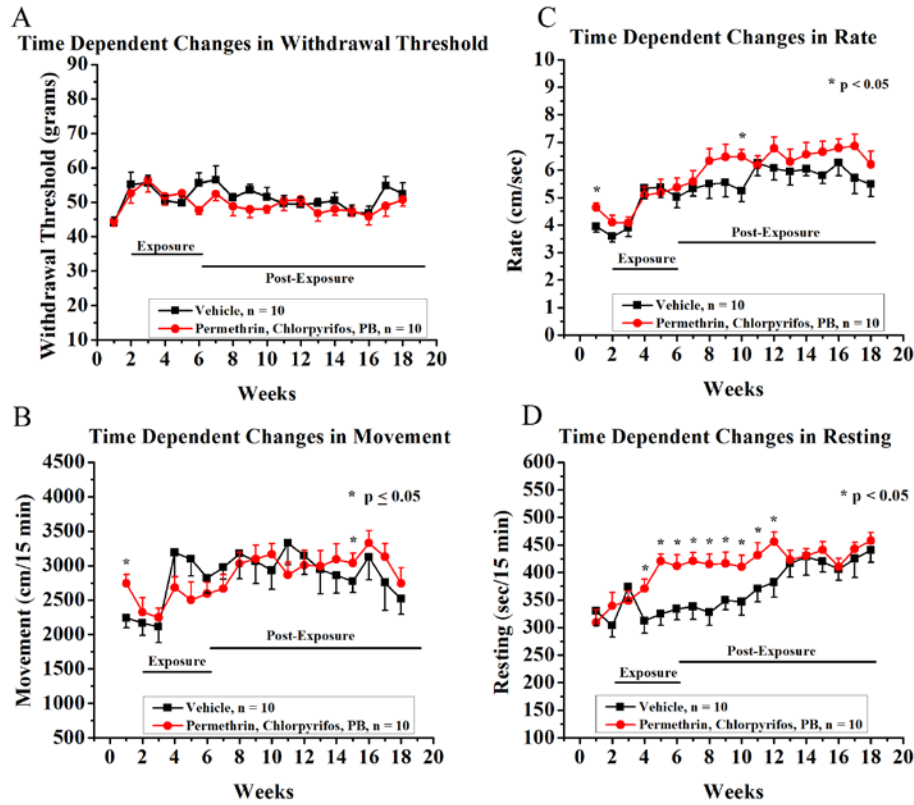


Figure 1. The Intensified 30 day NTPB Exposure Produced Some Lasting Behavioral Effects. Increasing the frequency of dosing (relative to Nutter and Cooper, 2014) for 30 days significantly increased resting times for up to 8 weeks post-exposure to GW chemicals (D, Week 12). Thereafter, vehicle exposed rats rested as much as those exposed to NTPB. None of the other behavior measures were consistently modified.

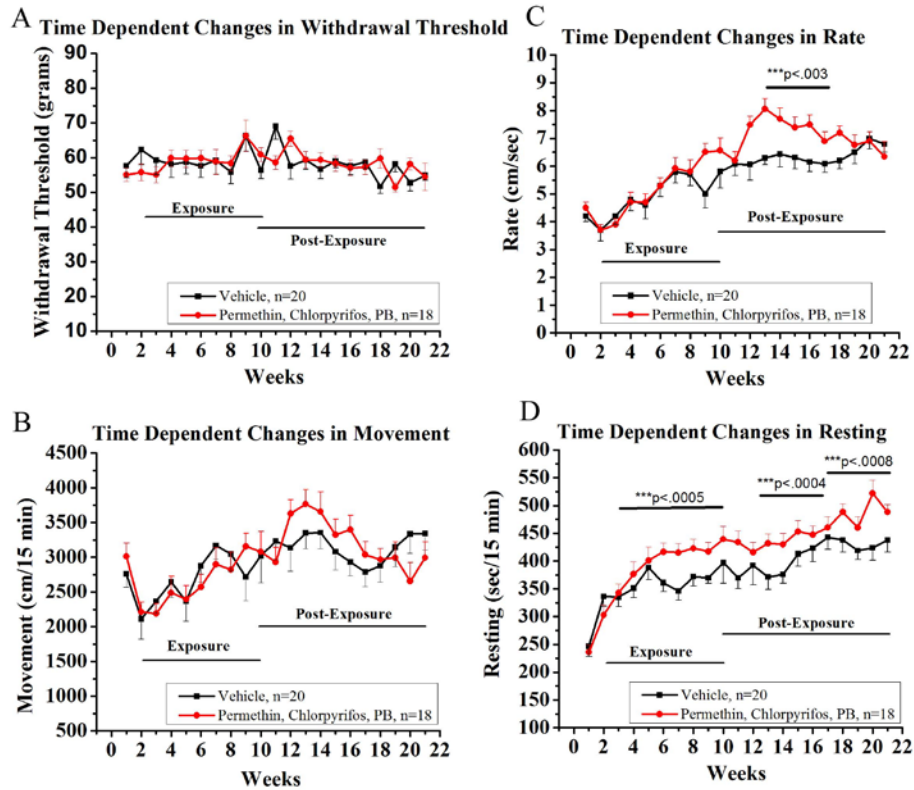


Figure 2. The Intensified 60 day NTPB Exposure Produced More Signs of Lasting Pain Behaviors. Increasing the frequency of dosing for 60 days significantly increased resting through 8 and 12 weeks post-exposure (D, Week 18 and 22 respectively). However, these rats moved faster during the 8 week post observation period (C, week 18). As a result, the evidence of a deep muscle or joint pain were mixed with this dosing procedure.

From the above data, it is clear that increasing the intensity of the chemical exposure could produce some persistent behavioral changes that outlasted chemical exposure by at least 12 weeks; however, this behavior was not entirely consistent with a chronic pain syndrome. We are encouraged by the persistence of some of these behavior changes, but it is clear that we need to further modify the exposure protocol.

Molecular Studies

Task 2: We will determine whether neurotoxicants/PB produce lasting changes in K_{DR} function that could contribute to chronic and widespread pain

Task 2a: Assess K_{DR} Voltage Dependent Activation 8 Weeks After Treatment.

Task 2b: Assess K_{DR} Voltage Dependent Activation 12 Weeks After Treatment.

Task 2c: Assess K_{DR} Voltage Dependent Deactivation 8 Weeks After Treatment.

Task 2d: Assess K_{DR} Voltage Dependent Deactivation 12 Weeks After Treatment.

Task 2e: Assess K_{DR} Normalized Amplitude 8 Weeks After Treatment.

Task 2f: Assess K_{DR} Normalized Amplitude 12 Weeks After Treatment.

Task 2g: Determine the K_v component Protein that Contributes to Changes in K_{DR} Amplitude, Activation and Deactivation.

Responsive to SOW Task 2, we examined the molecular impact of NTPB exposure on K_v channels that control cellular and axonal excitability. Eight and 12 weeks after NTPB exposure ended, vascular and muscle nociceptors were identified in DRG harvested from NTPB exposed and vehicle treated rats (Petruska et al., 2002). Total K_{DR} (absent K_v7) and the K_v7 current component of K_{DR} (isolated as a K_{DR} deactivation component; Task 2c, 2d and 2g) were isolated from other voltage dependent currents using conventional procedures (Nutter et al., 2013). We observed persistent changes in both K_v7 and K_{DR} currents following the new exposure protocol. Some of these changes predicted increased neural excitability and pain.

Consistent with previous reports on K_v7 currents, using the lower NTPB exposure levels (Nutter et al., 2013), the amplitude of the residual K_{DR} and K_v7 currents (normalized average and peak currents and conductances; Tasks 2e and 2f) were significantly increased 8 weeks following cessation of exposure (figure 3a and 3c; figure 4c). Both muscle and vascular nociceptors were affected by GW chemical treatments. These molecular shifts occurred while rats exhibited the unexpected combination of increased resting but faster movement during activity periods. By the time rats progressed to the 12 week post-test, interesting changes in behavioral and molecular

tests were observed. At 12 weeks post-exposure, K_{DR} and K_v7 currents were now significantly decreased in muscle nociceptors (figure 3d; figure 4b and 4d). This time period paralleled behavioral measures of significantly greater resting; rate and movement distance had normalized in treated rats by this time period. The changes in K_{DR} current amplitudes were accompanied by a hyperpolarizing shift in the voltage dependence at both 8 and 12 weeks (figure 5; Task 2b and 2c). These shifts were small ~1-3 mV and always in the hyperpolarizing direction.

As of this writing, some of the 12 week post-exposure data remains incomplete; a portion of the vascular nociceptor data is still being collected. Therefore manuscripts have not yet been submitted and interpretations must remain tentative. We will likely conduct action potential excitability studies to further determine the functional significance of these changes.

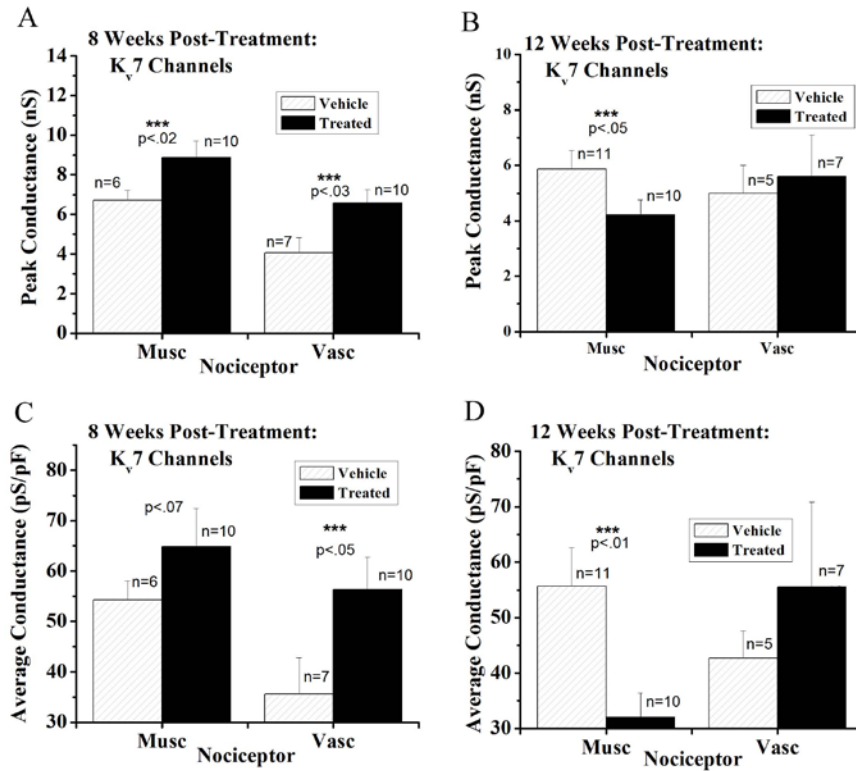


Figure 3. Altered Physiology of K_v7, 8 and 12 Weeks Following Exposure to Permethrin, Chlorpyrifos and PB (NTPB). K_v7 were affected at both 8 and 12 week post exposure periods. Decreases in peak and average conductance were most interesting at the 12 week post-exposure interval as these imply increased excitability. Data is still being collected in some of these studies.

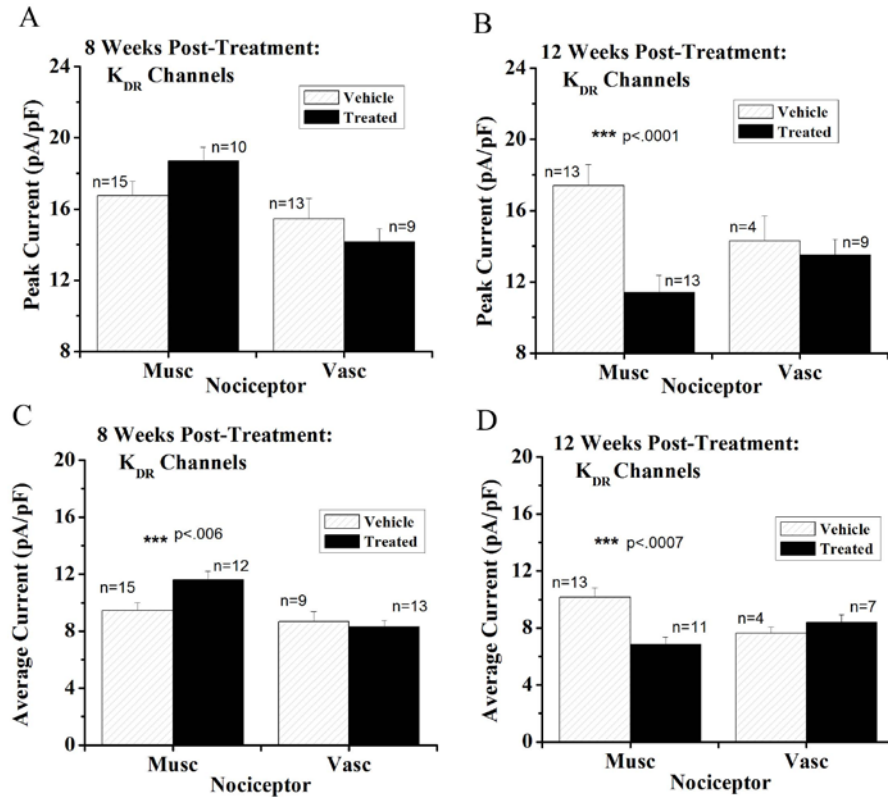


Figure 4. Altered Physiology of the residual Delayed Rectifier Channels (K_{DR}) 8 and 12 Weeks Following Exposure to Permethrin, Chlorpyrifos and PB (NTPB). The residual K_{DR} were affected at both 8 and 12 week post exposure periods. Decreases in peak and average current were most interesting at the 12 week post-exposure interval as these imply increased excitability. Data is still being collected in some of these studies. The residual K_{DR} is the K_{DR} current remaining after removal of the K_V7 component.

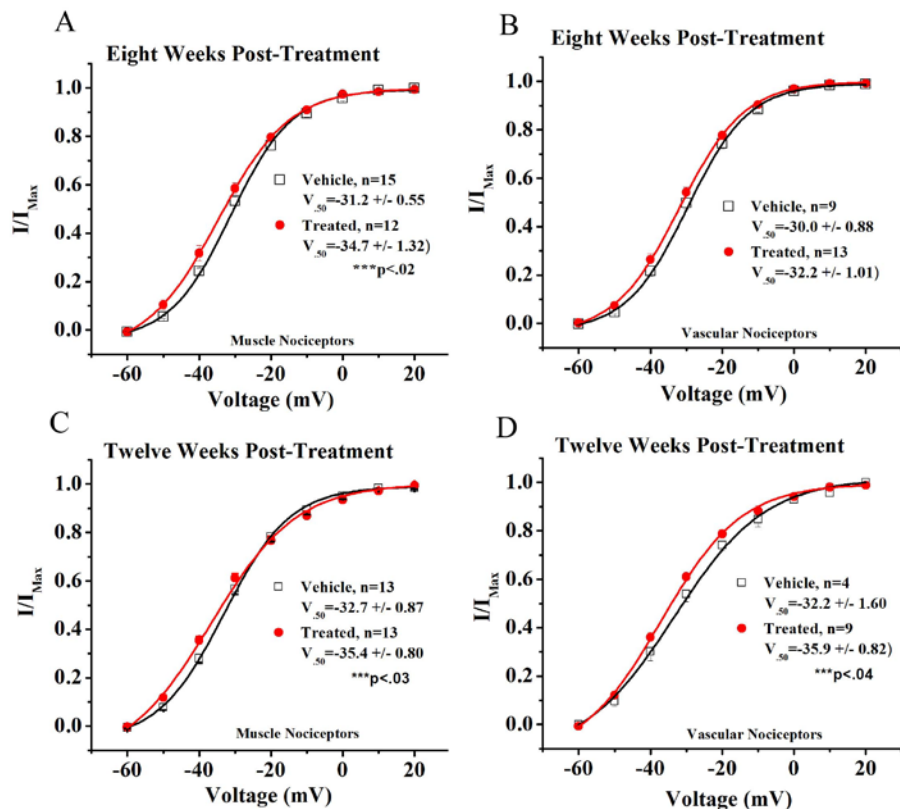


Figure 5. Voltage Dependence of Delayed Rectifier Channels (K_{DR}) 8 and 12 Weeks Following Exposure to Permethrin, Chlorpyrifos and PB (NTPB). Voltage constants (V_{50}) were hyperpolarized (leftward shift) at both 8 and 12 week post exposure periods. Data is still being collected in some of these studies. Note: Voltage constants were derived from Boltzmann fits to individual cells while the curves above reflect the average of all cells.

The total K_{DR} is a combination of a number of molecular subcomponents that differentially contribute to neuronal physiology. In DRG, the total K_{DR} current is a mixture of $K_v1.1$, $K_v1.2$, $K_v1.3$ and K_v7 channel proteins. The composition of the total K_{DR} in specific subtypes of DRG nociceptors (e.g., vascular, muscle) not well detailed (see Gold et al., 1996). We isolated the K_v7 component using the highly specific antagonist linopirdine. These findings were presented above. The K_{DR} data we report is actually the residual K_{DR} after the removal of the K_v7 contribution. For simplicity we refer to it as the K_{DR} . As part of Task 2g, we also attempted to separate the K_v1 components of the K_{DR} using K_v1 channel toxins (margatoxin; dendrotoxin K). These experiments were unsuccessful. The inability to separate the K_{DR} components is probably due the presence of mixed heteromers of the various K_v subunits. Therefore toxins specific to

homomeric channels composed of $K_v1.1$ or $K_v1.2$ or $K_v1.3$ might be ineffective against a heteromeric channel.

While we were unable to separate components of the K_{DR} using toxins, we were able to examine distinct contributions using a deactivation kinetic analysis. This analysis indicated that multiple components (at least 2) were likely to contribute to the residual K_{DR} . Exponential fits to the deactivation tails (at -30 mV) indicated the presence of a fast and a slow kinetic component that could represent distinct molecular entities (i.e., heteromers of $K_v1.1$ or $K_v1.2$ or $K_v1.3$). These components were differentially affected by exposure to GW chemicals (figure 6; Task 2c and 2d). The fast component showed little effect at 8 weeks post exposure, but was significantly increased at 12 weeks in both nociceptor classes (figure 6a and 6b). The slow component time constant of decay (τ) was more generally modified at both 8 and 12 weeks post exposure. We are still collecting data on the 12 week vascular nociceptor group. When this is completed (late October, 2014) we will submit this data for publication.

Taken together with the outcomes of experiments on K_v7 , the changes we observed to the fast and slow components of the K_{DR} indicate a broad influence of GW chemicals on several other K^+ channel proteins (potentially: $K_v1.1$, $K_v1.2$, $K_v1.3$). Although our measures are limited to nociceptors, these K_v ion channel proteins are widely present throughout the peripheral, central and autonomic nervous systems. They contribute both to neural excitability and the integrity of axonal coding of sensory and motor activity. Depending upon the site, pattern and character of these influences on neural activity, a wide variety of cognitive, sensory, autonomic and motor dysfunctions could be associated with K_v maladaptations.

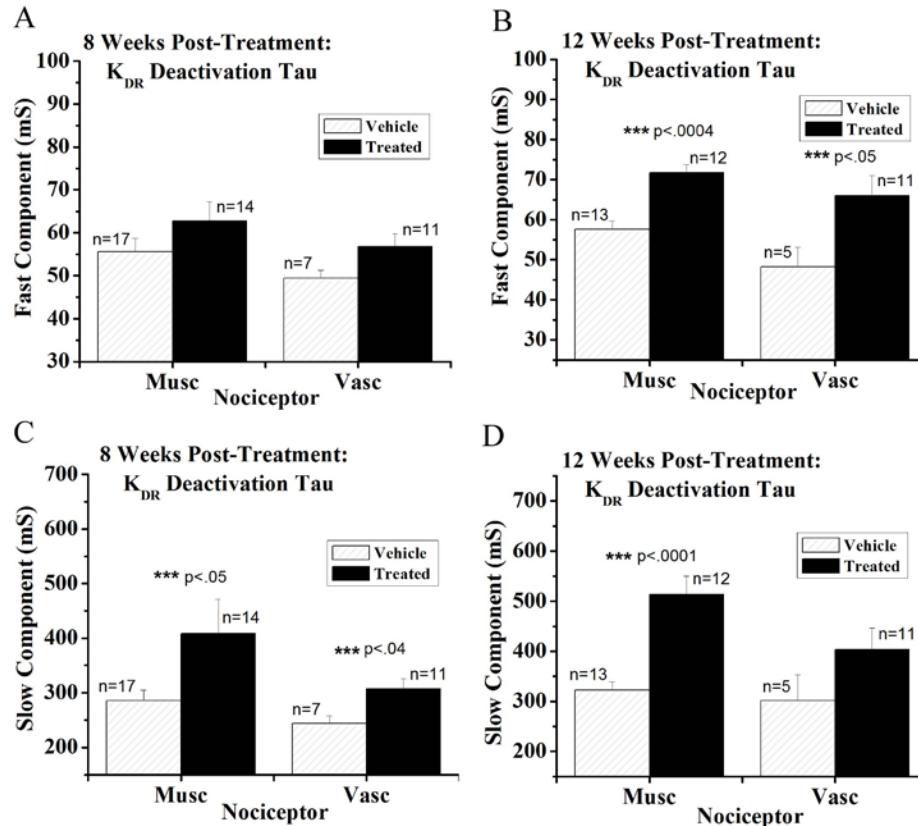


Figure 6. Deactivation Time Constants (Tau) were Increased by Exposure to GW Chemicals. A and B) 8 and 12 weeks after exposure the fast time constants trended longer, but only reached significance at 12 weeks. **C and D)** In contrast, significant changes in the slow decay constants were present at 8 and 12 weeks with some studies still underway (vascular nociceptors).

In summary, the increase in K_v amplitude and voltage dependence at 8 weeks post exposure suggested either a decline in neuronal excitability or an adaptation to oppose a strong depolarizing force. At 12 weeks post exposure, the K_{DR} current pattern inverted, exposing a significant decrease. The increased excitability that would accompany a decrease in total K_{DR} may be a component of the neural dysfunction following exposure to GW chemicals. Because behavioral changes were not entirely consistent with a chronic pain condition (increased resting but no significant change in movement or pressure-withdrawal threshold) certain pieces may still be missing from the puzzle. Pilot studies (funded by local resources) have suggested that DEET

may be an important GW chemical linked to persistent and broadly expressed pain behaviors. We have submitted this pilot data to the GWIRP as an Investigator Initiated Proposal.

Identify and Characterize Vascular Afferents

Task 3: To determine the anatomical, immunohistochemical, and electrophysiological properties of identified afferent neurons innervating the peripheral vasculature.

Task 3a: Determine electrophysiological properties of patched vascular DRG neurons labeled with DiI from peripheral arterial and venous targets in the tail and hindlimb.

Task 3b: Determine immunohistochemical phenotype for vascular DRG neurons in whole DRG sections (labeled as in Task 3a) using markers that differentiate Type 8 cells (e.g, NF-M, SubP, P2X3, IB4, ASIC1a, ASIC2a, KCNQ9).

Because alterations in the properties of vascular nociceptors have been linked to long term exposure to NTPB, we initiated a series of studies to better identify the family of vascular nociceptors and their specific properties. While muscle afferents have been specifically traced from muscle tissue, vascular afferents have been only assumed to innervate vessels because they are the only afferents traced from every tissue site examined (skin, muscle, viscera). In these studies (Task 3), we devised a new procedure to selectively trace vascular afferent neurons.

Several centimeters of the left tail vein of rats were isolated, a luminal plug placed at the cranial end, and the vein sutured closed at the caudal end. Two weeks following the injection of DiI-paste (a neuronal tracer), DRGs were excised, digested and dispersed cells were plated on 35 mm Petri dishes. Whole cell patch experiments were conducted on highly fluorescent neurons that represented those innervating the tail vein (Figure 7). Rats were examined post-mortem to confirm that DiI tracer had not leaked into surrounding tissue. Only cases without postmortem evidence of dye leakage outside the vascular compartment were accepted.

Following plating, highly fluorescent neurons were identified by fluorescent microscopy and targeted for whole cell patch experiments. After achieving the whole cell patch mode, classification procedures were applied and cell types were assigned to known and new classes

associated directly with vascular innervation. Consistent with our hypotheses, we identified traced neurons that fit the current signature classification of type 8 neurons that were identified following injections of skin, muscle, and viscera (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014; see Table 1; figure 8) and presumed to be vascular due to their universal anatomical representation. This confirms our hypothesis that type 8 neurons are vascular nociceptors, and that vascular nociceptor physiology is altered by NTPB. Two additional afferent classes were identified.

Table 1							
Physiological Signatures							
Type		Cell Size		Peak I_H		I_A Peaks	
		pF		pA/pF		number	
							IC Kinetics
							IC Base Width
							msec
8	n=7	70.42 +/- 6.4		0.76+/-0.23		4	slow
				p<.008			p<.0001
19	n=7	66.0+/-4.6		2.75+/-0.31		4	fast
				p<.0002			p<.02
20	n=8	75.1+/-6.9		7.22+/-1.80		4	fast
				p<.04			p<.0008
IC: Inward Current							

Table 1: Cell size and current properties associated with DiI traced vascular afferents. The amplitude of I_H currents and the width of inward currents (IC) form distinct signatures for these afferent types. Only type 8 neurons were nociceptive. 'P' values between measures indicate significant differences between adjacent values. 'P' values below type 20 measures reflect differences between type 8 and type 20.

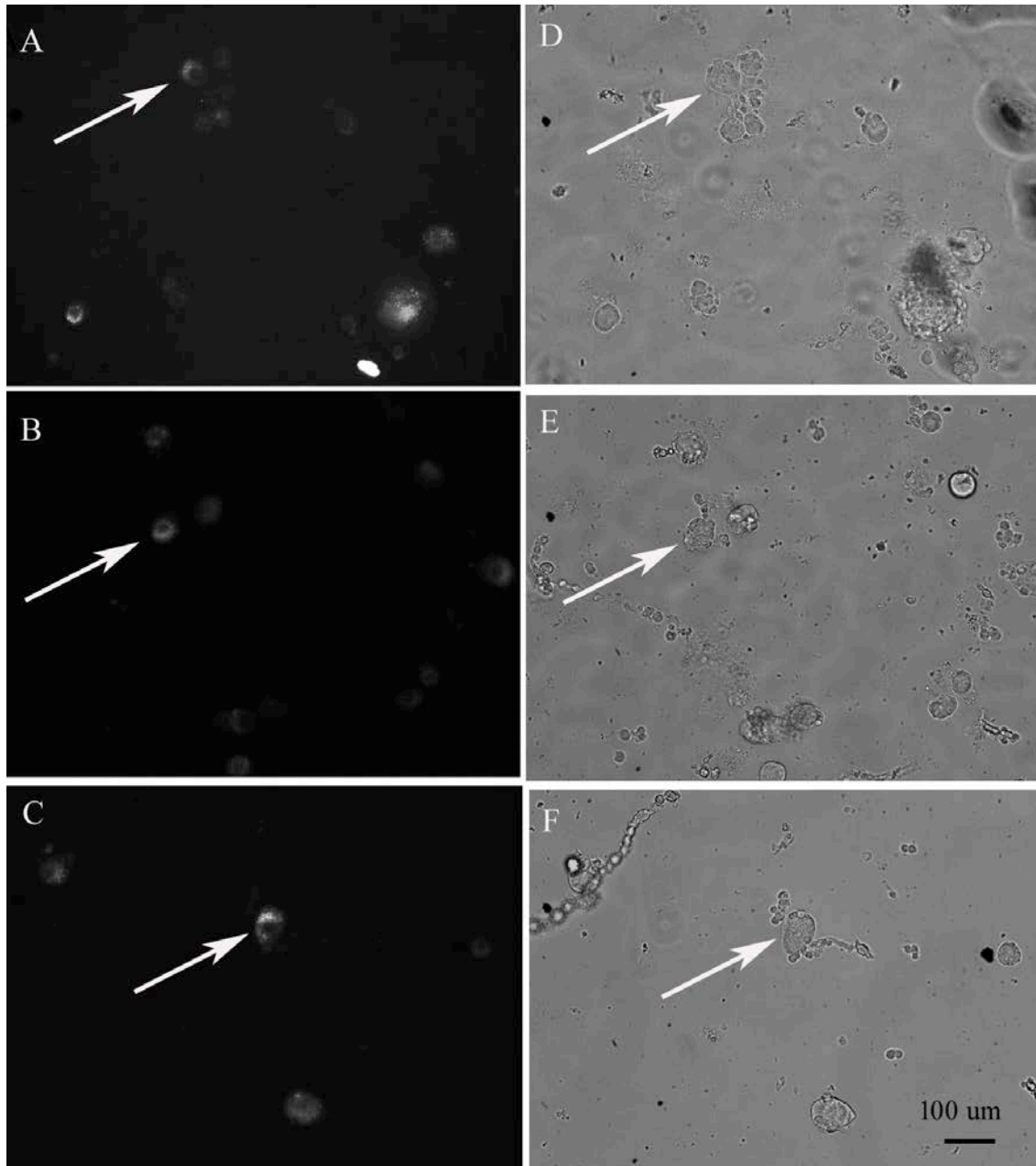


Figure 7. Di-I Fluorescent Labelled Vascular Afferents. A-C) Fluorescent Cells (arrows); D-F) Corresponding bright field images. Cells at arrows were patched and subsequently identified as type 8 (A, D), type 19 (B, E) and type 20 (C, F).

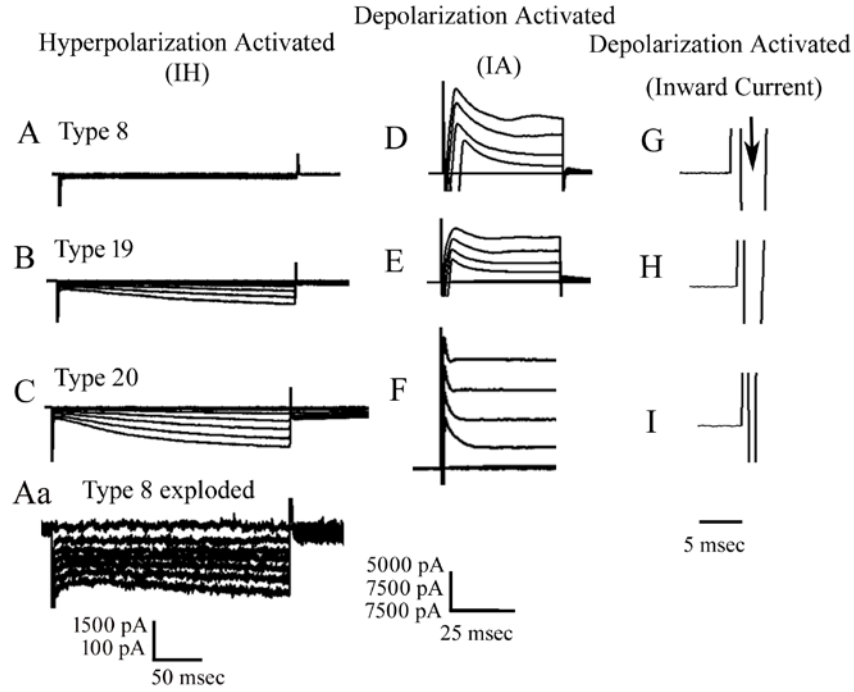


Figure 8. Physiological Signature Identifiers Associated with Vascular Afferents. **A-C)** Three phenotypes of vascular afferents differed by peak amplitude of hyperpolarization activated currents (I_H). **D-F)** Depolarization activated outward currents (I_A) peaks differed in kinetics but had similar voltage properties. **G-I)** Inward currents during depolarization exhibited significantly different width. See Table 1 for analysis. Classification procedures from Petruska (Petruska et al., 2002). Arrow indicates point of inward current width measurement. Aa) Exploded traces of panel 'A' shows small I_H . Vertical scale bars: A-C: 1500 pA; Aa: 100 pA; D-F: 5000, 7500 and 7500 pA respectively.

DiI traced vascular afferents were sorted by their physiological signatures. In order to identify important physiological characteristics of these neurons, they were exposed to ACh (500 μ M), capsaicin (1 μ M) and pH 6.0 solutions. Presentation of ACh and pH 6.0 were randomized. Capsaicin was always presented last in the sequence.

A portion of fluorescent vascular (tail vein) neurons had physiological signatures associated with type 8 neurons (figure 8). We had originally identified and characterized type 8 afferents in skin, viscera and muscle tissue using the DiI tracing technique (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014). The functional properties of vascular afferents we traced from the tail vein exhibited many of the same functional properties we observed in type 8 cells traced from other tissue sites; however a more complex pattern emerged than previously reported (Petruska et al., 2002; Rau et al., 2005). The functional characteristics resembled both type 8a and 8b

neurons: 1) capsaicin sensitive (CAPS); 2) fast (8a) or slow (8b) kinetic ASIC responders (Table 2, figure 9b and 9f; n=7); and 3) expression of neuropeptides CGRP and SP (substance P; figure 11).

Capsaicin sensitivity is a reliable marker of nociceptive function. The powerful capsaicin evoked currents of DiI traced vascular, type 8 neurons, are consistent with type 8 nociceptors in skin, muscle and viscera (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014). Other consistent properties indicate a high degree of proton sensitivity (ASIC-like responders) that would be required by afferents that monitor tissue pH (Jiang et al., 2006). In contrast, responses to ACh were mixed and exhibited some properties we had not previously reported. Most of the vascular nociceptor population exhibited irreversible baseline shifts consistent with a, muscarinic, G-protein coupled closure of a K_v7 current (figure 9c; Table 2). This is consistent with our observations that NTPB exposed nociceptors express modified K_v7 physiology (Nutter et al., 2013). Others vascular nociceptors (n=2) exhibited a slow desensitizing, nicotinic, inward current of the sort we have previously characterized in type 8 cells (Rau et al., 2005). These tracing studies are still incomplete and will continue into year 2. We should finish these studies in December, 2014.

Table 2							
Functional Properties							
Type		Capsaicin pA/pF		pH 6.0 pA/pF		ACh pA/pF	ACh kinetics
8	n=7	66.58+/-12.8		28.20+/-9.79	Desen: n=7	12.18+/- 8.81	Slow Desen: n=2
						2.04+/-0.79	Base Shift: n=5
				p<.02			
19	n=7	0		1.78+/-0.23	Non-Desen: n=6	29.6+/-6.6	Slow Desen: n=7
20	n=8	0		4.02+/-1.4	Non-Desen: n=7	16.8+/-6.3	Slow Desen: n=5
				p<.02			

Table 2: Chemical response properties of afferents traced from vascular sites. Only type 8 neurons were clearly nociceptive (capsaicin sensitive); although the lack of capsaicin sensitivity does not exclude nociceptive function. Application of pH 6.0 evoked distinct desensitizing (type 8) and non-desensitizing currents (types 19 and 20). Presentation of ACh (500 uM) evoked large slow desensitizing currents in non-nociceptive types 19 and 20, but irreversible base shifts in nociceptive type 8. 'P' values between measures indicate significant differences between adjacent values. 'P' values below type 20 measures reflect differences between type 8 and type 20.

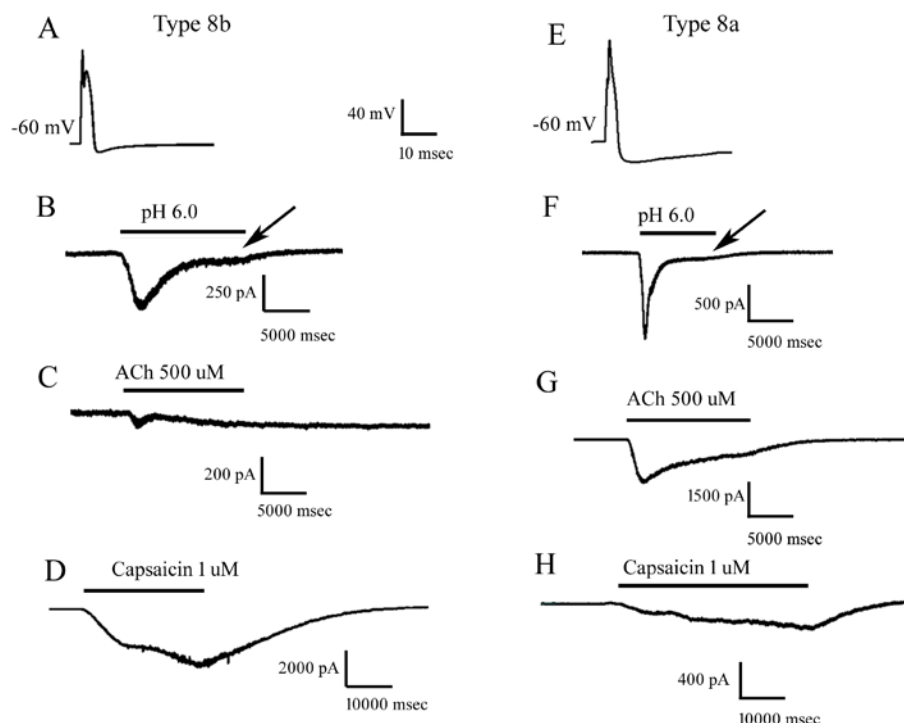


Figure 9. Chemical Response Characteristics of Vascular Nociceptors. **A-D)** Representative responses of type 8 vascular nociceptor to pH 6.0, ACh 500 uM and Capsaicin 1 uM. Five traced afferents exhibited this pattern. Note that the response to ACh (C) is not a true evoked current as the trace never returns to baseline. A baseline shift usually indicates the closing of a resting K^+ current, but that has not been verified by experiment. **E-H)** Representative responses of a type 8 vascular nociceptor to pH 6.0, ACh 500 uM and Capsaicin 1 uM. Two traced afferents exhibited this pattern. Note the powerful nicotinic currents of this group (G) that differed from those in 'C' when ACh was applied. Horizontal bars indicate the duration of chemical application. Arrows point to a non-desensitizing component of the response to pH 6.0.

In our tracing studies, we discovered additional classes of vascular afferents that were distinguished from type 8 by their signature identifiers (Table 1; figure 8), as well as by their distinct functional properties. We have designated these as types 19 and 20. The functional properties of these two classes did not differ from each other (figure 10), so it is not certain that the distinction between type 19 and 20 (Table 1), is a worthwhile distinction. Still, many additional properties of these afferents, yet to be discovered, may prove them to be functionally as well as physiologically distinct. Capsaicin insensitive (CAPI) type 19 afferents (n=7) and type 20 (n=8) afferents both responded to pH 6.0 with small, non-desensitizing, $K2p$ -like currents (Table 2; figure 10b and f), as well as powerful nicotinic responses to ACh (figure 10c and 10g). We are scheduled to examine the properties of these $K2p$ currents in the second year of the project (Task 1). Capsaicin reactivity was absent (D and H). It should be noted that late,

non-desensitizing, currents could also be observed at pH 6.0 in nociceptive type 8 cells when the strongly desensitizing component had fully desensitized (figure 9B and 9F). These might represent K2p currents known to be expressed in vascular nociceptors (Rau et al., 2006).

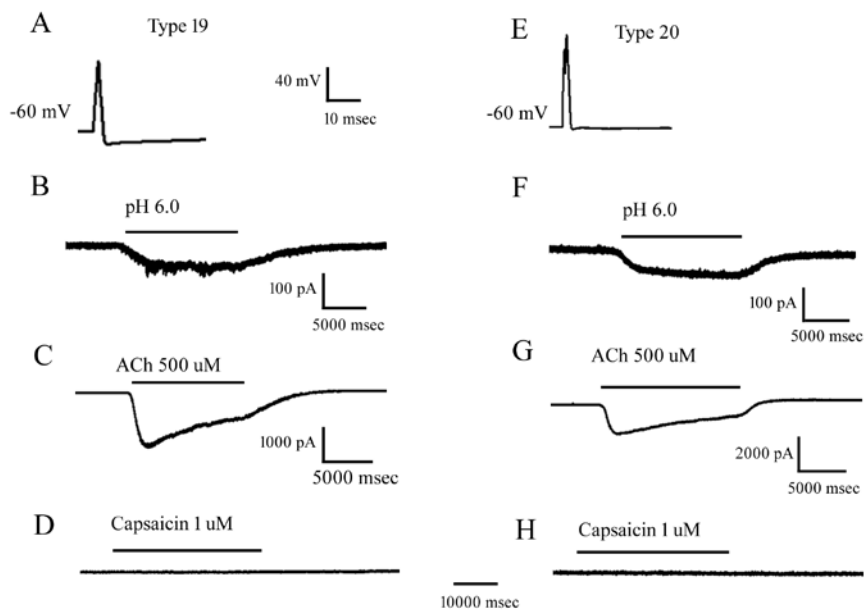
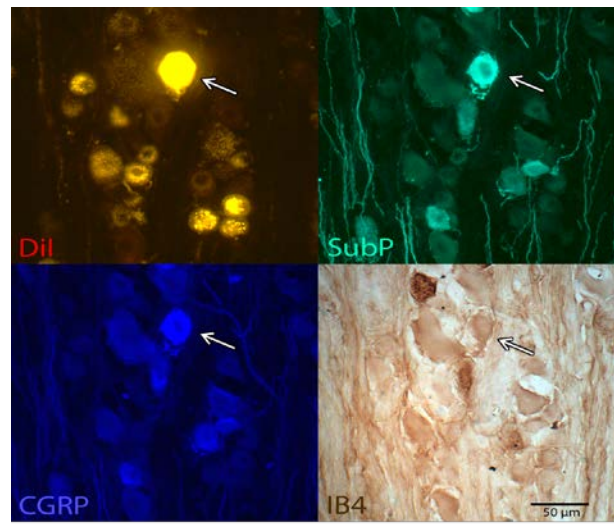


Figure 10. Chemical Response Characteristics of Non-Nociceptive Vascular Afferents. **A-D)** Representative responses of a type 19 vascular nociceptor to pH 6.0, ACh 500 uM and Capsaicin 1 uM. Seven traced afferents exhibited this pattern. **E-H)** Representative responses of a type 20 vascular nociceptor to pH 6.0, ACh 500 uM and Capsaicin 1 uM. Eight traced afferents exhibited this pattern. Within the limits of our experiments, these two classes of neurons exhibited identical chemical response characteristics. Horizontal bars indicate the duration of chemical application.

Additional tracing studies examined the immunocytochemical properties of vascular afferents (Task 3b). In these experiments, DiI tracer was injected in the same manner as previously described. Rats were sacrificed and whole DRG were excised and sectioned. DiI labeled vascular afferents were identified in whole DRG sections. Multiple staining procedures were used to determine the immunocytochemical phenotypes of these neurons. As shown in figure 11, vascular afferents were mainly found in the medium sized cell body range, consisted of both myelinated and unmyelinated groups and expressed peptides CGRP and/or SP. This profile fits with properties previously published on the characteristics of type 8 cells (Rau et al., 2007; Rau et al., 2014).



Vascular DRG neuron exhibiting SP+ /CGRP+ /IB4-

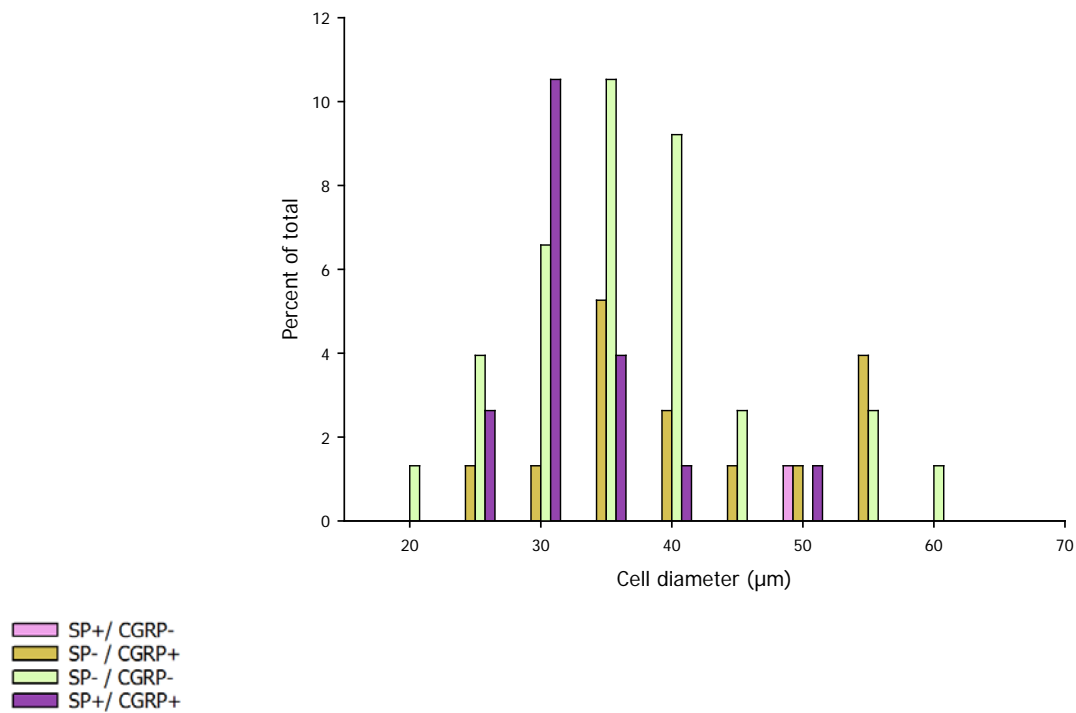


Figure 11. Immunohistochemical (IHC) and Morphometric Data from Vascular Afferents. Data from cryosections of the L6 DRG. Top panel has four views of the same section under different fluorescence filters/light showing a DiI-labeled cell (white arrow) in L6 containing positive IHC markers for SP and CGRP, but negative for IB4. Bottom panel represents a cell diameter plot of 56 DiI-labeled vascular afferent cells, in L6, processed with the same series as in the top panel, and measured with ImagePro software. Note the significant numbers of CGRP+ cells, with roughly half negative for SP.

Discussion

Tracing Studies

Vascular nociceptors play an important role in migraine headache (Harriott et al., 2009; Zhang et al., 2013) and arthritic joint pain (Suri et al., 2007; Ashraf et al., 2011). They also contribute to other forms of tissue pain due to their widespread representation and critical role in autonomic reflexes and tissue perfusion. Moreover, vascular nociceptors express the vasoactive and pro-inflammatory peptides Substance P and CGRP (CGRP; calcitonin gene related peptide; Petruska et al., 2002; Rau et al., 2014). These vasodilating neuropeptides are released as paracrine messengers in the region of the vascular endothelium. They set into motion the synthesis and/or release of a number of pro-inflammatory agents (prostacyclin, histamine, nitric oxide (NO)) from nearby cells to produce classic neurogenic inflammation (Geppetti et al., 2005; Benemei et al., 2009). Vascular nociceptor derived CGRP has been shown to play a key role in the genesis of migraine headache pain (Durham, 2010); not only through peripheral actions on vessels, mast and synovial cells but also in the CNS, where central projections of vascular nociceptors release CGRP onto central nervous system neuroglia prompting the production of cytokines (IL-1 β , IL-6) chemokines (CCL2), NO and other pro-inflammatory agents (Li et al., 2008; De Corato et al., 2011; Malon et al., 2011).

Our tracing studies confirmed that vascular nociceptors are affected by exposure to GWI chemicals. In addition, we have identified two additional subtypes of vascular afferents. Although these are not nociceptors by conventional characteristics, any misregulation of tissue perfusion could contribute to a chronic pain state. Misregulated vascular reflexes could lead to poor responses as tissue oxygen requirements vary. As a result, tissue pH could drift from normal to acidic levels and thereby activate muscle and vascular nociceptors through normal processes. Muscle and vascular nociceptors express the highly proton sensitive ASIC channels that are activated by pH as high as 6.8 (Jiang et al., 2006; figure 9).

Behavioral Studies. We have demonstrated that exposure to GW chemicals can modulate certain important cellular surface proteins that participate in the control of neuronal excitability (Na_v1.9, K_v7). Due to the widespread distribution of these channels, such shifts in their physiological properties could have wide ranging implications for the development of many of the symptoms

of GWI. At the same time, other important channels were relatively unaffected by exposure to PB, chlorpyrifos and permethrin ($\text{Na}_v1.8$; Nutter et al., 2013). Some of the molecular changes (i.e., increase activity of $\text{Na}_v1.9$) seemed to be clearly associated with increased neuronal excitability (and thereby a chronic pain state; Nutter and Cooper, 2014) while others would actively oppose such excitability shifts (K_v7 ; Nutter et al., 2013). Importantly these modulations extended beyond the period of NTPB exposure by at least 8 weeks. In our previous reports, there was little indication that lasting behavioral changes accompanied any of these molecular adaptations to GW chemical exposure.

In this year's effort, we tried to improve the behavioral model, in part, to get a better idea of the relationship of membrane channel adaptations to the development and maintenance of behavioral indices of a delayed chronic pain. Our first attempt was to intensify the exposure by increasing the frequency of application of GW chemicals while maintaining the same dosing level. After a 30 day exposure to the intensified protocol, some increased resting was observed out to 6 weeks post exposure. When the intensified protocol was extended to 60 days, shifts in resting behavior was maintained out to 12 weeks post exposure. Resting is a common sign of pain in animals. There were, however, no signs of decreased pressure-pain thresholds and some indications that movement rate increases occurred (at 8 weeks post) that were inconsistent with pain at the 8 week delay period. Importantly, the counterintuitive movement rate increases returned to normal levels at the 12 week delay while resting duration remained significantly elevated. Accordingly, the behavioral model was significantly improved by increasing the dose frequency, but some further adjustment of doses and exposure set may yet be indicated. Pilot experiments using DEET in addition to permethrin, chlorpyrifos and PB seemed to greatly improve the behavioral outcome. Significant behavioral changes in pain threshold, movement and resting were observed out to 16 weeks when DEET was added to the protocol (Appendix figure A1, page 42). It should be noted that all the required control groups have yet to be completed in this pilot study (funded by internal sources).

Molecular Studies. The behavior at the 8 week delay contained mixed indications of a persistent painful condition. Shifts in molecular activity in muscle and vascular nociceptors, at this time, could be related to these behavioral changes. During this period, we observed increased activity of both K_{DR} and K_v7 channels in the NTPB chemically exposed rats (Nutter et al., 2013). The

broad shifts in K^+ channel activity, that were observed, were an appropriate adaptation for the opposition of a strong depolarizing force (hyperpolarized $V_{.5}$; increased amplitude or conductance; increased deactivation time constants). Consistent with a recently published report, we replicated the finding that this strong depolarizing force could be an increase in $Na_v1.9$ activity (Nutter and Cooper, 2014). Because the disparate movement rate increases at 8 weeks had diminished, the 12 week delay data gave the best total picture of a delayed chronic pain state. The molecular changes during this time might be particularly relevant. While we do not have the full set of molecular assessments at the 12 week window, we did observe a compelling shift in the pattern of molecular changes during that specific time period. Specifically, at the 12 week post exposure delay, the amplitude of the K_{DR} and K_v7 currents shifted from a significant increase to a significant decrease. Both of these reversals are consistent with the development of a chronic pain state; particularly if the strong depolarizing influence of $Na_v1.9$ was still present. Because some of the molecular data is still being collected we cannot draw any conclusions at this time. We will have completed the collection of this data by mid November. We will submit this data for publication at that time. Unfortunately we do not have any data on the status of $Na_v1.9$ at 12 weeks post exposure with the intensified dosing schedule. The $Na_v1.9$ data should be further pursued although it is not part of the existing SOW.

We also need to further develop the behavioral model that includes DEET exposure in addition to the NTPB procedure we currently use with improved outcomes. We have submitted a proposal to fully pursue the DEET component in behavioral, molecular and therapeutic studies.

4. Key Research Accomplishments

- Exposure to GW Chemicals Modulates K_{DR} Channel Activity 8 and 12 Weeks Post-Exposure
- Exposure to GW Chemicals Modulates K_v7 Channel Activity 8 and 12 Weeks Post-Exposure
- Altered Channel Activity Occurs in Both Vascular and Muscle Nociceptor Phenotypes
- Traced and Characterized Vascular Nociceptors Exhibit Currents with K_v7 Current-like Properties
- Non-Nociceptive Vascular Afferents Were Identified and Exhibited $K2p$ -like Currents
- Intensified Exposure to PB, Permethrin and Chlorpyrifos Produces Some Pain-like Behaviors 12 Weeks Post-Exposure

5. Conclusion:

We have now shown that an intensified exposure to GW chemicals (PB, permethrin, chlorpyrifos) can produce pain-like behaviors that last at least 12 weeks after exposure. Although all indices of pain were not congruent, the persistence of the effect on resting behavior is encouraging. Some additional efforts along the lines of the present approach may bring about a more complete pain syndrome with properties fully suggestive of myalgia and arthralgia. By this we mean not only increased resting, but also reduced movement and perhaps decreased muscle pain-pressure thresholds. Based upon recent pilot studies, we plan to add DEET to the exposure group.

In cellular studies, we have confirmed the identity of vascular nociceptors and produced further data that their physiology is modulated by GW chemical exposure. The functional properties of this afferent pool were characterized in greater detail. Based upon previous investigations of the type 8 phenotype, the powerful ASIC-like currents we observed were not surprising (Jiang et al., 2006). However, the lack of a nicotinic response component in a sub-phenotype was surprising (Rau et al., 2005). It appeared that a portion of the vascular nociceptor pool exhibits mainly M-current like reactivity (K_v7) when challenged with ACh. The known modulation of M receptors by GW chemicals is noteworthy (Abou-Donia et al., 2002; Abou-Donia et al., 2003; Abou-Donia et al., 2004).

In addition, our cellular studies have identified two more vascular afferent phenotypes. Their contribution to the syndrome will be investigated in year 2. Vascular sourced pain could arise directly from vascular nociceptors or indirectly via a deficiency in vascular reflex regulation of blood flow. These reflexes are essential for maintaining good tissue perfusion and thereby normal tissue pH. Vascular reflex disturbances have been observed in GW veterans (Haley et al., 2009; Liu et al., 2011; Li et al., 2011; see also Ojo et al., 2014). If tissue becomes acidic, pain arises through normal events processed by an extensive pool of vascular, muscle and joint nociceptors that are highly sensitive to tissue pH (Jiang et al., 2006).

At the molecular level, we observed that K_{DR} proteins expressed in vascular and muscle nociceptors exhibited long lasting changes in their activity following exposure to GW chemicals.

In our previous studies, using a less intense dosing schedule, we observed similar increases in the activity of K_v7 (a K_{DR} subtype) and $Na_v1.9$ proteins that persisted for 8 weeks (Nutter and Cooper, 2013; Nutter et al., 2014). We have now replicated changes in the physiology of $Na_v1.9$ and K_v7 with the intensified protocol and in the presence of some positive behavioral outcomes. With the new dosing procedure, some of these molecular adaptations now persist out to 12 weeks after the termination of chemical exposure (K_{DR} , K_v7). Because we do not have a full picture of each molecular adaptation at each time point in the presence of a fully pathologic behavioral outcome, it is not possible to draw a definitive picture of the critical pattern of molecular responses to exposure to GW chemicals associated with delayed persistent pain. It is clear that this group of chemicals does produce lasting changes in important voltage sensitive membrane proteins that regulate neuronal excitability. As we go forward, we will try to fill these gaps. In particular we should pursue changes that occur to $Na_v1.9$ at 12 weeks post exposure. At present we believe that $Na_v1.9$ is a key driving force for aberrant activity in both vascular and muscle nociceptors. Heightened activity of $Na_v1.9$ indicates increased excitatory drive on vascular and muscle nociceptor pools. The observed shifts in K_{DR} and K_v7 oppose these excitability shifts. However at 12 weeks post-exposure, when pain behaviors were maximal, the opposition by K_{DR} and K_v7 activity had dissipated or reversed sign. Under such circumstances, the unopposed drive of $Na_v1.9$ could produce conditions favorable to a widespread chronic pain in muscles and joints. We have not yet examined whether the up-regulation of $Na_v1.9$ physiology persists at 12 weeks post exposure when the K_v physiology that opposes it has inverted or diminished. As we alter the dosing methods and improve the behavioral outcomes, we also produce mismatches between prior discoveries made in similar but not identical chemical exposures. Filling these gaps creates natural replications and strengthens our confidence in the relationship between molecular events and chemical exposures. If we confirm that a normalized K_v7 physiology creates an imbalance between excitatory and inhibitory drives on deep tissue nociceptors, we have identified a point of attack for therapeutic agents. FDA approved drugs that increase K_v7 activity and reduce $Na_v1.9$ activity provides an opportunity to test this interpretation.

6. Publications, Abstracts, And Presentations:

A. Peer Reviewed Scientific Articles:

In Preparation

B. Abstracts and Presentations

Cooper, B.Y., Nutter, T.J., Dugan, V.P., Johnson, R.D. Classification and characterization of vascular afferents in the rat. An abstract accepted by the Society for Neuroscience, Washington, D.C. November, 2014.

7. Inventions, Patents and Licenses

none

8. Reportable Outcomes:

- 1) An intensified dosing procedure that exposed rats to permethrin, chlorpyrifos and pyridostigmine bromide produced some lasting pain-like behaviors in rats that outlasted the chemical application period by 12 weeks.
- 2) Behavioral effects were accompanied by increased activity of muscle and vascular nociceptor expressed K_{DR} proteins that function to limit repetitive discharge during AP initiation at the peripheral ending and further to ensure the integrity of axonal relay of sensory information.
- 3) Vascular nociceptors sensitive to GW chemicals were shown to belong to a family peptidergic neurons that express paracrine, pro-inflammatory, mediators CGRP and SP, the highly proton sensitive ASIC channels; and exhibit K_{V7} -like membrane current shifts (M-current) in response to ACh.
- 4) Additional, non-nociceptive, phenotypes of vascular afferents were identified that might contribute to a disturbance of vascular reflex regulation.

9. Other Achievements:

Based, in part, upon data collected during this funding period, we applied for new funding from GWIRP:

Neurovascular and Autonomic Dysfunction Associated with Gulf War Illness Pain

GW140066

We received an invitation to submit a full proposal. This proposal was submitted on September 25th 2014.

If awarded, this funding would start Sept. 29, 2015

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11. Appendices

Supplementary Figures

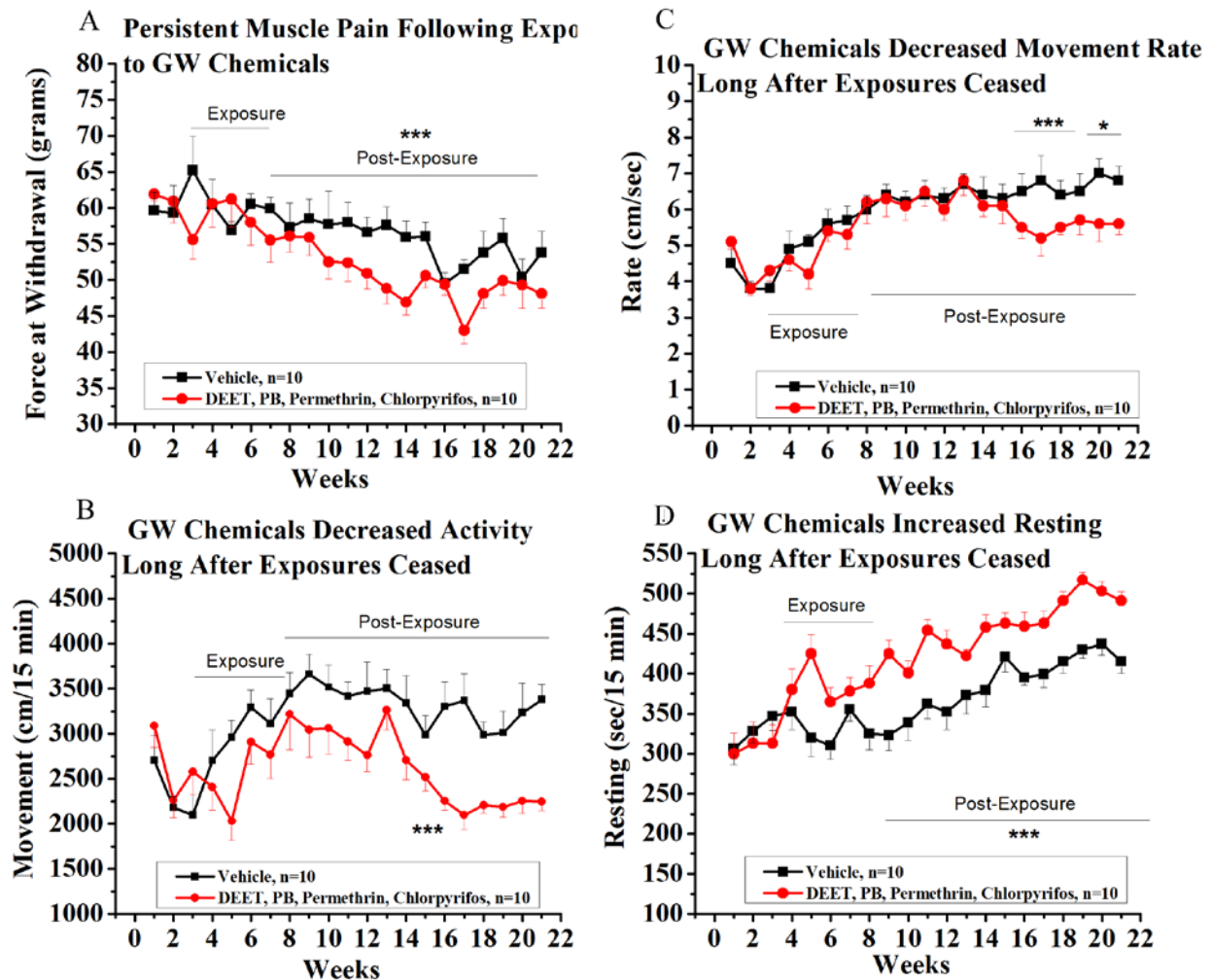


Figure A1. Adding DEET to the Exposure Protocol Produces Signs of Persistent Pain Behavior Effects.

Adding DEET to the intensified 30 day exposure produced a broad range of pain behaviors for up to 16 weeks post exposure. Decreased pressure threshold withdrawal, decreased movement distance, increased resting periods and slower movement rate were all consistent with deep muscle and joint pain. All tests were blinded to the experimenter or collected by an automated activity box. * $P < .05$; *** $P < .001$

Methods

Exposure Protocol. Juvenile male rats weighing between 90 and 110 g were used in all studies (Sprague-Dawley; Harlan). Over a period of 60 days, 16 rats were exposed to permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), and pyridostigmine bromide (PB; 13 mg/kg). Permethrin, in ETOH, was applied every day to a shaved area of the back between the forelimbs. Chlorpyrifos was administered by a subcutaneous injection (corn oil) once every 7 days. PB was given daily by oral gavage (tap water). The latter represented a standard military dose of PB (assuming a 70 kg body weight). Twenty (20) additional rats received only vehicle exposures using an identical administration schedule. Rats were sacrificed for electrophysiological studies 8 and 12 weeks after chemical exposures had ended. All rats underwent behavioral testing before, during and after chemical exposures (see below). There was little indication that chemical exposures affected body weight. The average body weights of vehicle and chemically exposed rats did not differ at the 8 week post-exposure period. 442.4 +/- 5.0; n=20, Vehicle; and 430.8 +/- 8.6 g. n=16, Exposed; p<.28). Rats were weighed once per week throughout the studies and doses were adjusted accordingly.

All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO.

Assessment of Pain Behaviors

On arrival, rats were acclimated to the behavioral procedures for 2 weeks before dosing began. Testing continued throughout the entire dosing and post-dosing periods. Pressure pain withdrawal thresholds were measured using a computer monitored, hand held test device (PAM; Ugo Basile). Pressure was applied via a 5 mm diameter ball force transducer to the semitendinosus and biceps femoris muscles (right hind limb). During force application, the applied pressure was monitored and displayed to the experimenter on a video screen. Video feedback enabled the rate of force application to be regulated according to a visual standard. When the rat withdrew its limb, the force at withdrawal was automatically registered and stored. To complement pressure pain testing, activity levels (movement distance, movement rate, and rest times) were recorded automatically by infrared sensors in an activity box (15 min test

period). Behavior tests were conducted on both chemically exposed (permethrin, chlorpyrifos, PB) and vehicle treated (ETOH, corn oil, water) animals over an identical time course. Rats were tested once per week on the behavioral tasks. Most tests were conducted in 'blinded' conditions. Only 'blinded' testing scores were used in the analysis.

Electrophysiological Studies

Preparation of Cells. Dorsal root ganglion neurons (DRG) were harvested from chemically and vehicle exposed rats 8 and 12 weeks after termination of chemical exposures. Rats were anesthetized (Isoflurane) and rapidly euthanized by decapitation (Harvard Instruments). The spinal column was removed, bisected and the DRG were dissected free from T11 to S1. Ganglia were trimmed, cut into strips and digested in Tyrode's solution containing collagenase A (4 mg/ml) and Dispase II (10 mg/ml; Roche Chemical). A 10 ml centrifuge tube containing the dissected ganglia was placed in a heated, shaking water bath for 90 minutes at 35° C (EDVOTEK Digital Shaking Water Bath). Gentle trituration was then used to break up visible strips of ganglia. The dispersed neurons were then digested for an additional 45 minutes, and then spun at 1000 Hz (30 sec). The supernatant was discarded. The remaining pellet was dispersed into 2 ml of Tyrode's, tritured and plated on 8-10, 35 mm, polylysine coated, Petri dishes (Fluorodish). Plated neurons were bathed continuously in a Tyrode's solution, containing (in mM) 140 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. All electrophysiological studies were conducted at room temperature (21 ° C) within 10 hours of plating. Only one cell was used per Petri dish. Electrodes were formed from borosilicate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). For studies on K⁺ channels, the pipette solution contained (in mM): 120 KCl, 5 Na₂-ATP, 0.4 Na₂-GTP, 5 EGTA, 2.25 CaCl₂, 5 MgCl₂, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

Recording and Characterization of Muscle and Vascular Nociceptors. Whole cell patch clamp recordings were made with an Axopatch 200B (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and Digidata 1322A (Molecular Devices). Series resistance (R_s) was compensated 65-70% with Axopatch

compensation circuitry. Whole cell resistance and capacitance were determined by the Clampex software utility. Recorded currents were sampled at 10-20 kHz and filtered at 2 kHz (Bessel filter).

Once the whole cell mode was achieved, neurons were classified as type 5 (muscle) or type 8 (vascular) nociceptors using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2000; 2002; see also Xu et al., 2010; Ono et al., 2010). Anatomical targets of type 5 and type 8 neurons were determined by a series of anatomic tracing experiments (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014). Cells not fitting the classification criteria were discarded.

Isolation and Characterization of K_{DR} and K_v7 Channel Currents.

K^+ currents were characterized in a solution containing (in mM) 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-amino pyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with KOH. The pipette solution contained (in mM): 120 KCl, 5 Na_2 -ATP, 0.4 Na_2 -GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. The osmolarity was approximately 290 mOsm.

K_{DR} Currents. For the purpose of this study, the K_{DR} current was defined as the residual total K^+ current following removal of the K_v7 component. Attempts to remove other components using K_v1 inhibitors Margatoxin or Dendrotoxin K were unsuccessful. The voltage dependent activation of the residual total K_{DR} current, was assessed, as a tail current, after a 3 minute application of the K_v7 inhibitor linopirdine (10 μM). From a holding potential of -60 mV, a 2000 msec conditioning pulse (-100 mV) was followed by 11 consecutive command steps to 20 mV (10 mV increments; returning to -60 mV). The amplitude of the tail current at -60 mV was measured from the peak relative to the baseline current recorded 2500 msec after repolarization. For each recorded neuron, the amplitude of tail current was normalized to the peak evoked current and then plotted against the activation voltage to obtain a current-voltage relationship. A Boltzmann function was fit and a $V_{1/2}$ determined for each individual cell. Student's T-test was used to compare the pooled $V_{1/2}$ values for vehicle and GW chemical treated cells. To assess average and peak amplitude, the K_{DR} tail currents, at each voltage, were normalized for cell size

(current amplitude (pA) divided by the cell size parameter (pF)). These normalized amplitudes were averaged across functional activation voltages (-50 to 0 mV) to obtain a mean current amplitude. The normalized peak current was determined by inspection. Student's T-tests were performed on peak and average amplitude as described above. The alpha level was set at .05.

The kinetics of deactivation of K_{DR} tail currents were assessed using current traces evoked at -30 mV ($V_{.50}$). A time constant for deactivation (τ_{deact}) was determined by fit of a function of the form: $A_1 \exp(-(t-k)/\tau_1) + A_2 \exp(-(t-k)/\tau_2) + C$, to the falling phase of the current trace over a range representing points from 90% of the peak to 10% of the base current. It was determined that the K_{DR} deactivation tail current were best fit by a two parameter model that included a slow time constant (τ_1) and a fast time constant (τ_2). The time constants for each neuron were pooled for fast and slow components. Student's T-tests were performed as described above on the vehicle treated and GW chemical exposed cells. The alpha level was set at .05.

K_v7 Current. A current subtraction method was used to isolate K_v7 mediated currents from other K^+ currents that were present as deactivation tail currents. The cell size normalized peak and average K_v7 current was assessed as a conductance to eliminate deactivation voltage confounding of the peak current. For the K_v7 deactivation protocol: a 1000 ms step command to -20 mV was followed by a series of repolarizing 10 mV steps from -10 to -90 mV (1000 ms; $V_H = -60$ mV) followed by a return step to -60 mV. A tail current could be measured during the repolarization steps. The K_v7 voltage deactivation protocol tests were first applied in the K^+ isolation solution containing .001% ETOH (pre-applied for 2 min). This was followed 2 minutes later by application of the K^+ isolation solution containing the K_v7 specific antagonist linopirdine (10 μ M in ETOH; 3 min application). The K_v7 voltage deactivation protocol was reapplied. The linopirdine sensitive K_v7 current was isolated by subtraction.

The amplitude of the linopirdine sensitive tail current was measured from a point beginning 10 ms after the repolarizing voltage step (-30 to -90 mV) to the point 10 ms prior to the return step to -60 mV. The cell size normalized amplitudes (pA/pF) were converted into a conductance (G). A mean G was computed over the range of functional deactivation steps (-30 to -70 mV) to obtain a mean normalized conductance. The peak conductance was determined by inspection.

Tracing Vascular Afferents. Several centimeters of the left tail vein of rats were isolated, a luminal plug placed at the cranial end, and the vein sutured closed at the caudal end. Two weeks following the injection of DiI-paste, DRGs were excised, digested and dispersed cells were plated on 35 mm Petri dishes. Whole cell patch experiments were conducted on highly fluorescent neurons that represented those innervating the tail vein. Rats were examined post-mortem to confirm that DiI tracer had not leaked into surrounding tissue. Only cases without postmortem evidence of dye leakage outside the vascular compartment were accepted. Fluorescent and bright field images were taken using the NIS Elements Software package (Nikon Instruments). Cells were observed through a 20X Plan Fluor objective (0.45 NA). Fluorescence was captured by a RET-2000R camera (Quantitative Imaging; Melville, NY).

Immunohistochemistry. Immunohistochemical and morphometric studies of vascular afferents were performed in serial cryosections. DiI applied to the vascular endothelium and wall of the lateral tail vein was transported to the sensory neuronal cell bodies in the DRG. After 13 days of tracer transport time, the animals were euthanized and perfused transcardially with 4% paraformaldehyde. The left and right L5-S2 DRGs were dissected free, post-fixed in 4% paraformaldehyde overnight, and cryoprotected in a 30% sucrose solution until equilibrated. DRGs were embedded in OCT compound (Baxter), serially sectioned on a cryostat at a thickness of 14 μ m, and thaw-mounted on alternating poly-L-lysine double-subbed slides. Nucleated DiI-positive DRG neurons were directly visualized via fluorescence microscopy and digitally imaged with Zeiss Axiophot/ImagePro software. Slides were immunohistochemically processed for various cellular markers. For example, to detect neurofilament-M (NF-M) in the DRG cell bodies, a marker for myelinated neurons, mouse anti-neurofilament-M antibody, diluted 1:500, was used. The neurofilament-M primary antibody was labeled with the fluorescent secondary antibody anti-mouse-350 (blue), diluted 1:100. A second primary/secondary process to detect CGRP with anti-rabbit primary (1:200) and anti-rabbit-488 (green) was performed on the same slide. Alternate slides were similarly processed with the same fluorophore to detect substance P (SP) in the cell bodies, using mouse anti-substance P antibody diluted 1:200. All primary antibodies will be applied to the tissue for 12-18 hours, and secondary antibodies were applied for 4 hours. After quantitation and digital storage of the images, we detected binding to the lectin IB4 (a marker for cells with unmyelinated axons) as follows: incubation for 12-18 hours in biotinylated lectin IB4 followed by an avidin-biotin complex for 1 hour prepared at half

concentration (Elite standard Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and detected using DAB (diaminobenzidine) as the chromogen (20 mg in 50 mls PBS; Sigma). Following application of DEPEX mounting media, the slides were coverslipped and the Di-I labeled cells re-imaged, identified as positive or negative for IB4, and their diameters/circumferences measured with morphometric ImagePro software tools. The section thickness of 14 μm and exclusion of cell images that are not nucleated, or those which fell on the border of the image, will avoid the double counting of the cells.

Abstracts Published/Presented

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Classification and characterization of vascular afferents in the rat

Introduction. A series of studies have revealed that current signature classified afferents exhibit tissue specific innervation and distinct expression patterns of protein subunits mediating nicotinic (α_7 , $\alpha_3\beta_4$; $\alpha_3\beta_4\alpha_5$), protonergic (ASIC1-3), heat sensing TRP (V1, V2), and 2 pore potassium channels (K2p; TASK-1, 2, 3). In contrast, the type 8 nociceptor could be traced from multiple tissues (hairy skin, glabrous skin, muscle, distal colon, penile mucosa). We have recently shown that type 8 neurons exhibit persistent molecular alterations following an 8 week exposure to insecticides linked to Gulf War Illness (Nutter and Cooper, 2014). We hypothesized that type 8 neurons might also be tissue specific, but represent a class of nociceptors that innervate tissues common to all injection sites. In the present studies we examined whether type 8 could be traced specifically from venous tissues.

Methods. Experiments were conducted on young adult male Sprague-Dawley rats (n=6). Several centimeters of the left tail vein were isolated, a luminal plug placed at the cranial end, and the vein sutured closed at the caudal end. Two weeks following the injection of DiI-paste, DRGs were excised and plated on 35 mm Petri dishes. Whole cell patch experiments were conducted on highly fluorescent neurons. Only cases without postmortem evidence of dye leakage outside the vascular compartment were accepted. Following current classification procedures (Petruska et al., 2002), neurons were exposed to ACh (500 μ M), capsaicin (1 μ M) and pH 6.0 solutions.

Results. Both known and previously unidentified type 8 neurons were labeled by DiI. Many vascular afferents manifested characteristics of kinetically distinct type 8a and 8b neurons (capsaicin sensitive (CAPS), fast and slow kinetic ASIC responders; n=7). Capsaicin insensitive (CAPI) type 8 afferents (n=7) exhibited significantly higher H-current (4.1 +/- 1 vs 1.3 +/- .5 pA/pF; p<.02), smaller cell size (59.5 +/- 2.4 vs 78.3 +/- 5.5; p<.02) and narrower action potentials (4.7 +/- 0.2 vs 8.1 +/- .6 msec; p<.0003). CAPI type 8 neurons responded to pH 6.0 with small, non-desensitizing, K2p –like currents (127 +/- 24.2 pA; n=7). Two distinct cholinergic response forms were represented in CAPS neurons: slow desensitizing, $\alpha_3\beta_4\alpha_5$ -like currents (n=4), and irreversible holding current shifts suggesting closing of a resting current (156 +/- 52 pA; n=3).

Conclusions. Type 8 nociceptors innervate vascular tissue. These include previously identified type 8a and 8b CAPS responders and a new type 8c CAPI subclass. Cholinergic response patterns suggested further specializations among vascular afferents.